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Ultrastructural Localization of Specific Gonococcal Macromolecules with Antibody-Gold Sphere Immunological Probes

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In an effort to determine the ultrastructural location of specific macromolecules on the surface of intact microorganisms and in experimentally infected tissues, a new method of rapidly conjugating antibodies to gold spheres via a staphylococcal protein A intermediary has been developed. This new technique provides the excellent density of marking and versatility of sphere size provided by existing gold methods, but decreases preparation time, decreases the chance of bacterial contamination of antibody reagents, and increases specificity of marking. Staphylococcal protein A-coated gold spheres were conjugated with antibodies from rabbits immunized with purified gonococcal pili. The resulting gold-antibody conjugates allowed demonstration of antibody binding to pilus structures of the same gonococcal strain whose pili were used to raise the antibody and demonstration of the lack of antibody recognition of pilus structures on two other gonococcal strains. The failure of gold spheres to attach to isogenic nonpiliated clones of the homologous gonococcus indicated the absence of pilus antigens on the surface of these organisms. The use of a double label—small gold spheres conjugated to anti-pilus antibody and larger gold spheres conjugated to anti-protein I antibody—allowed the simultaneous localization of two gonococcal antigens.

In an effort to prevent bacterial infections, vaccine candidates are actively being sought among an ever-increasing number of isolated and purified bacterial surface components (e.g., proteins, lipopolysaccharides, pilus peptide fragments, and capsular carbohydrates) (5, 8, 26, 27). Likely to be of greatest promise as vaccine candidates are molecules that (i) play a role in the disease process, (ii) are accessible on the surface of the microorganism for interaction with host cells, and (iii) are accessible to antibodies acquired after vaccination. As a first step in assessing various surface structures with regard to their accessibility for interaction with host cells and with antibodies, we evaluated several electron-dense markers that, when linked to antibodies such as those which might be elicited by vaccines, would reveal the location of the antigen in electron microscopic preparations. The presence of the marker on the surface of bacteria exposed to an antibody-marker preparation would indicate the accessibility of the target antigen to host cells and to vaccine-elicited antibody.

Previously described techniques for linking electron-dense markers to antibodies or for using such complexes were found to be unsatisfactory for various reasons. By substantially modifying existing immunoelectron microscopic methods (10, 21), immunological probes were constructed which consisted of electron-dense gold spheres linked to antibodies through a protein A intermediary. The attachment of antibody directly to gold sphere-protein A (GpA) complexes and exposure of bacteria to these conjugates resulted in adequate marking of target antigens with minimal background marking. This method proved to be rapid and productive of immunological probes that made it possible to determine the accessibility of antigens on intact microorganisms and to determine the presence or absence of cross-reactive antigens in other strains of the same species of bacteria. This paper provides details of the method and the results of studies in which gold sphere-antibody conjugates were used to assess the immunological cross-reactivity of pili among gonococcal strains and the presence or absence of pilus antigens on the surface of homologous nonpiliated gonococci.

MATERIALS AND METHODS

Microorganisms. The microorganisms used were T2 (piliated) and T4 (nonpiliated) transparent clones of *Neisseria gonorrhoeae* R10, a T2 (piliated) transparent clone of *N. gonorrhoeae* MS11, and a T2 (piliated) slightly opaque clone of *N. gonorrhoeae* MEL. Organisms were frozen in defibrinated sheep blood at −70°C until the day before use. At this time samples were thawed, cultivated on a solid medium consisting of gonococcal agar base (Difco Laboratories, Detroit, Mich.) containing 2% (vol/vol) IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and placed in a 2% CO_2 incubator at 37°C. After 18 to 20 h of incubation, colonies were harvested for immunoelectron microscopic studies of surface structures.

Buffers and media. Phosphate-buffered saline was prepared by dissolving 27.4 g of Na_2HPO_4 (Sigma Chemical Co., St. Louis, Mo.) and 7.88 g of $NaH_2PO_4 \cdot H_2O$ (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 1 liter of distilled, deionized water to make solution A. Solution B was 8.5% NaCl in water. The final phosphate-buffered saline solution consisted of 40 ml of solution A, 100 ml of solution B, and 860 ml of water. The final pH was adjusted to 8.0 with 1 N NaOH (J. T. Baker).

Stabilizing buffer consisted of 500 mg of Carbowax 20-M (Union Carbide, South Charleston, W.V.) per liter of 0.15 M NaCl and 0.05 M Tris-hydrochloride (Trizma base; Sigma) with pH adjusted to either 7.0 or 8.0. All solutions were passed through a membrane filter (0.45 μm pore size; Nalge Co., Rochester, N.Y.) before use.

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The medium for suspension of microorganisms and dilution of albumin was 0.05 M HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (Sigma)-buffered Eagle's minimal essential medium (MEM) pH 7.45, containing Earle salts and L-glutamine (GIBCO Laboratories, Grand Island, N.Y.) (HEPES-MEM).

Antibody. Polyclonal anti-R10 pilus antibody was prepared in 5-lb (ca. 2.3-kg) New Zealand white rabbits. Fifty micrograms of pilus protein (purified as previously described [25]) in 1 ml of phosphate-buffered saline was emulsified with an equal volume of complete Freund adjuvant and administered by multiple subcutaneous and intramuscular injections. Booster injections in Freund incomplete adjuvant were administered 6 weeks later. Ten days thereafter, the animals were bled by cardiac puncture, and the sera were filter sterilized and stored at 4°C.

Monoclonal anti-protein I antibody was prepared and purified as previously described (29).

Preparation of GpA complexes. Solutions containing 100 ml of water and either 2 ml of 1% (wt/vol) sodium citrate (for gold spheres of 18-nm diameter) or 1 ml of 1% (wt/vol) sodium citrate (for gold spheres of approximately 40-nm diameter) were brought to a boil in acid-cleaned Erlenmeyer flasks by using reflux as previously described (9, 10, 14–16, 22). To these solutions were added 0.7 ml of a 1% (wt/vol) solution of chloroauric (III) acid trihydrate ($HAuCl_4 \cdot 3H_2O$; Fisher Scientific Co., Fair Lawn, N.J.). Gentle boiling for 30 min resulted in a color change from purple to red to orange. This last color change indicates the reduction of gold and formation of spheres, the size of which is governed by the ratio of the citrate to chloroauric acid (9). The suspensions were allowed to cool to room temperature, and the pH was adjusted to approximately 7.0 with 0.2 M potassium carbonate by using pH-sensitive test tape.

The total amount of protein (protein A in this case) optimal for coating the gold spheres contained in 40 ml of the above suspension was determined in the following manner. A solution containing 2.5 mg of a salt-free lyophilized staphylococcal protein A (Sigma) per ml of sterile water for injection (Abbott Laboratories, North Chicago, Ill.) was diluted 1:10 in sterile water for injection, and a series of twofold serial dilutions was made, ranging in final dilution from 1:10 to 1:1,280. To 0.1 ml of each of these serial dilutions in a small test tube, 0.5 ml of the gold suspension was added. The mixture was vortexed and then incubated for 5 min at 25°C. At the end of this period, 0.5 ml of 10% (wt/vol) NaCl was added to each tube, and the least amount of protein (the highest dilution) that coated the gold spheres so as to prevent flocculation by the NaCl was noted. The range of dilutions that included this endpoint was recognized by a change in color of the mixture from red (excess protein) to blue (inadequate protein). The endpoint dilution was determined spectrophotometrically as the last dilution before a decrease of 10% or greater in the optical density measured at 520 nm. The usual optical density of those tubes having excess protein (red color) ranged from 0.35 to 0.40. The concentration of protein A in the endpoint dilution was calculated, and a 10% excess was added to that figure. This figure (the endpoint concentration plus 10%) was used to determine the amount of the original protein A solution (2.5 mg/ml) that was diluted to 8 ml for combination with the 40 ml of gold sphere suspension. The GpA suspension was incubated for 5 min at 25°C in Nalgene ultracentrifuge polycarbonate tubes. The GpA suspension was stabilized further with 4 ml of 1% (wt/vol) Carbowax 20-M (13).

After the incubation and stabilization with Carbowax, the suspensions of GpA were centrifuged at 28,000 × *g* (17,500 rpm) for 1 h (18-nm gold spheres) or 20 min (40-nm gold spheres) at 4°C in a Beckman L5-50 Ultracentrifuge (16). After the supernatant, which contained unattached protein A, was discarded, the pellet was suspended in 40 ml of stabilizing buffer (pH 7.0) with aggitation on a Vortex Genie mixer at maximum amplitude for 10 s. This left a small, dense red button in the tube, which was not further disturbed. The same tube was centrifuged as above. The supernatant was removed and 20 ml of stabilizing buffer (pH 7.0) was added to suspend the pellet as before. The resulting GpA stock solution was stored in siliconized glass vials (Wheaton Scientific, Millville, N.J.) at 4°C. The GpA complexes showed no apparent instability or loss of affinity for immunoglobulin G (IgG) during 6 months in storage.

Before each lot of GpA complexes was used and periodically thereafter, it was tested for its ability to bind the Fc portion of IgG by using the hemagglutination of human IgG (anti-D)-coated human erythrocytes as an indicator (21). These cells (Reagent Red Blood Cells, Ortho Coombs Control 3% cell suspension; Ortho Diagnostic Systems, Raritan, N.J.) were incubated with serial 10-fold dilutions of GpA complexes in 1% albumin in stabilizing buffer (pH 8.0). Normal human erythrocytes without attached antibody (Reagent Red Blood Cells 3-5% Suspension, Spectrogen I or II; Biological Corp. of America, West Chester, Pa.) that were incubated with GpA dilutions were used as a negative control, whereas IgG-coated human erythrocytes incubated with dilutions of rabbit anti-human IgG were used as a positive control (anti-human serum [rabbit]; Ortho Diagnostic Systems). Each incubation was performed simultaneously at room temperature in a microtitration rounded multiwell plate (Linbro Scientific Inc., Hamden, Conn.). Hemagglutination with definite dilution endpoints was seen with anti-human serum or GpA complexes incubated with IgG-coated erythrocytes. No agglutination was observed when GpA complexes were incubated with normal human erythrocytes.

Attachment of antibody to GpA complexes. GpA complexes (100 μl) were placed in 5- by 20-mm cellulose propionate tubes (Beckman Instruments Inc., Palo Alto, Calif.) and ultracentrifuged at approximately 50,000 × *g* for 2 min (Beckman Airfuge). The supernatant was discarded, and 100 μl of gonococcal pilus antiserum or gonococcal protein I antibody (1:100 in stabilizing buffer pH 8.0) was added to suspend the pellet. The mixture was incubated with mild aggitation (Tektator shaking platform, 100 rpm) for 30 min at 25°C. The preparation was then centrifuged at approximately 50,000 × *g* for 2 min to separate the unbound antibody from the GpA-antibody conjugate. As much supernatant as possible was removed from the resulting pellet, and 150 μl of stabilizing buffer (pH 8.0) was added to suspend the conjugate. The preparation was centrifuged as before, and a second wash was performed in an identical manner with final suspension of the GpA-antibody conjugate in 1% bovine serum albumin in stabilizing buffer (pH 8.0).

Preparation of electron microscopy specimens. Organisms of *N. gonorrhoeae* were prepared and negatively stained in the following manner (20). Briefly, the organisms were transfered from the surface of agar plates to a drop of HEPES-MEM by using a platinum wire loop and gentle aggitation. Copper electron microscopy specimen grids (200 mesh; Ted Pella Inc., Tustin, Calif.), which were coated with polyvinyl Formvar (Electron Microscopy Sciences, Fort Washington, Pa.) and carbon stabilized, were floated Formvar side down on the suspensions of the organisms. After 1 min the grids were transferred to drops of reagents in the

following sequence (duration of incubation at room temperature is given within parentheses): (i) HEPES-MEM (brief wash); (ii) 1% bovine serum albumin in HEPES-MEM (15 min); (iii) 1% bovine serum albumin in stabilizing buffer, pH 8.0 (brief wash); (iv) 18-nm GpA–anti-gonococcal pilus antibody conjugate (30 min). The grids were then held by the rim with forceps and agitated in a beaker containing PBS (pH 8.0) for 30 s and then floated on 1% phosphotungstic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) in distilled water (pH 6.0) for 1 min. Between each step, the edges of the grids were held against filter paper to remove residual fluid. When performing double-labeling experiments, a second 30-min incubation with 40-nm gold spheres conjugated with anti-gonococcal protein I antibody was interjected after step iv. After flotation on phosphotungstic acid, the grids were placed in separate Beem capsules (Ted Pella) and allowed to air dry (Lab-Line Dessicab; Lab Line Instruments Inc., Mellrose Park, Ill.).

Examination of electron microscopy specimens. All specimens were examined with a Siemens Elmiskop IA transmission electron microscope (Siemens Corp., Berlin, West Germany), and photographs were processed on Polycontrast rapid II RC or Kodabrome paper (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

This technique involving GpA-antibody conjugates in studies of surface structures of intact microorganisms was examined by testing its ability to (i) differentiate gonococcal pili from other proteins in the gonococcal outer membrane, (ii) determine the degree of antigenic cross-reactivity of pili from different gonococcal strains, (iii) determine the presence or absence of immunoreactive pilus subunits on the surface of gonococcal organisms from an isogenic nonpiliated strain, and (iv) differentiate the physical location of two different gonococcal antigens.

Marking of pili with GpA-antibody conjugates. Gonococci were negatively stained with phosphotungstic acid and examined by electron microscopy. Pili were seen as interwoven linear strands emanating from the edge of the organism or lying free on the surface of the Formvar grid. *N. gonorrhoeae* strain R10 was exposed to anti-R10 pilus antibody conjugated to gold spheres as described above. Large numbers of GpA–anti-pilus antibody conjugates were seen adjacent to pili (Fig. 1A). Virtually no gold spheres were seen attached to either outer membrane blebs, the surface of the organisms, or the Formvar background. Thus, the GpA–anti-pilus antibody conjugates appeared to be specific in their recognition of pili as distinct from other surface structures of the gonococci.

Marking of pili of other gonococcal strains with GpA-antibody conjugates. *N. gonorrhoeae* strain MS11 was exposed to the same GpA–anti-R10 pilus antibody conjugate preparation as was used with strain R10. There was no significant marking of pili, the organisms, or the Formvar background (Fig. 1B). Similar results were obtained with another piliated gonococcal strain, MEL. Therefore, the GpA–anti-pilus antibody conjugate was specific in its differentiation of homologous and heterologous pili.

Marking of nonpiliated gonococci. When nonpiliated R10 gonococci from type 4 colonies were exposed to the GpA–anti-R10 pilus antibody conjugate there was no attachment of gold either to the organisms or the outer membrane blebs (Fig. 1C). These results again indicate the ability of the complexes to distinguish pili from other gonococcal surface structures and suggest that nonpiliated gonococci do not contain pilus antigens in any form on their surface.

Simultaneous marking of two gonococcal antigens. When R10 gonococcal organisms were exposed in sequence to small (18-nm) GpA–anti-R10 pilus antibody conjugates and large (40-nm) GpA–anti-protein I antibody conjugates, the large gold spheres marked the surface of the gonococcus, and the small gold spheres marked pili (Fig. 2). This technique allows the simultaneous localization and differentiation of two individual antigenic surface structures on bacteria.

DISCUSSION

Since the development in 1973 of a reliable technique for producing gold spheres ranging in diameter from 15 to 150 nm in diameter (9), these spheres have been used increasingly as markers for indicating the location of macromolecules in specimens examined by light, transmission electron, or scanning electron microscopy. One of the major uses of these spheres has been in demonstrating antigen detection by antibody (2, 11–14, 16, 17, 24). There are two basic methods for detecting antigens on cell surfaces or in tissue sections with gold spheres: a direct and an indirect method. The direct method consists of placing specific antibody directly onto the surface of gold spheres and using the resultant gold-antibody complexes to detect the target antigen (7). In the indirect method cells or tissue sections are exposed to specific antibody, and the location of bound antibody is detected with a subsequent incubation with GpA complexes (21).

The direct method has greater specificity of marking when compared with the indirect method (13, 14). However, placing antibody directly onto the surface of gold spheres requires dialysis, which is time consuming and poses a risk of microbial contamination.

The indirect gold marking method most frequently used involves GpA complexes to detect antibody affixed to its target antigen. This indirect method is limited to use with those antibody types, primarily IgG, that bind to staphylococcal protein A (6, 18). We initially attempted to mark gonococcal pili by the indirect method (1, 3, 4, 19, 21, 23) by exposing gonococci to various dilutions of anti-pilus antisera and detecting bound antibody with GpA complexes. Despite multiple trials, including the use of albumin in the reagents to block background marking, our attempts were plagued by apparently nonspecific attachment of gold spheres to the electron microscope grids. The use of the indirect method was also attended by diminished resolution of pili, apparently because of attached antibody.

The above problems were circumvented quickly and easily by contructing the immunological probe so that the antibodies were attached to the GpA complexes. When these GpA-antibody conjugates were used, there was heavy marking of pili and negligible appearance of the marker in the background or in association with outer membrane blebs or other nonpilus gonococcal structures. Thus, the specificity of marking was dramatically improved by using protein A as an intermediary between the gold spheres and the antibodies.

We have developed this technique with rabbit antisera raised against purified gonococcal pili. The GpA-antibody conjugates marked pili from the homologous organism, but not pili from two heterologous strains. Thus the GpA-antibody conjugates were capable of demonstrating antigenic heterogenity among pili from different gonococcal strains. Since the pilus preparation used to immunize the rabbits contained the portion of the pilin subunit that appears

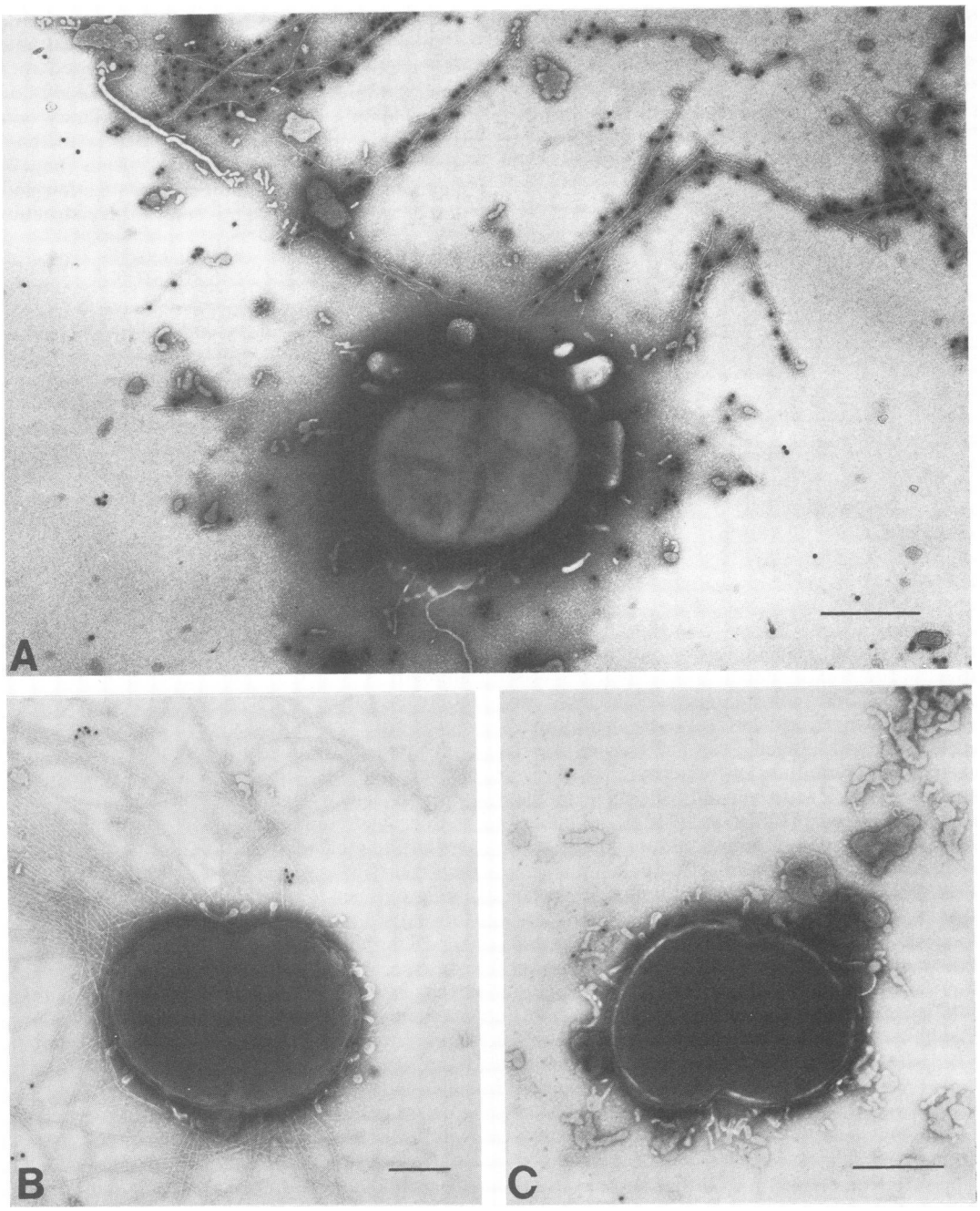

FIG. 1. *N. gonorrhoeae* exposed to gold spheres conjugated with antisera against purified pili from gonococcal strain R10. (A) *N. gonorrhoeae* R10, a colony type 2 (piliated) transparent clone. Note the attachment of the gold sphere-antibody conjugates almost exclusively to the pili and not to outer membrane blebs or the surface of the organism. (B) *N. gonorrhoeae* MS11, a colony type 2 (piliated) transparent clone. Note absence of association of the gold sphere-antibody conjugates with the pili of this heterologous gonococcal strain. (C) *N. gonorrhoeae*, R10, a colony type 4 (nonpiliated) transparent clone. Note the absence of gold sphere-antibody conjugates on the surface of this homologous nonpiliated R10 gonococcus suggesting the absence of pilus antigens in any form. Negative stain. Bars, 0.5 μm.

common to the pili of all gonococcal strains and is immunogenic in a purified form (the CB-2 fragment [25, 26]), the absence of marking of the heterologous pili suggests that the antiserum lacked antibody against the common antigen, that the common antigenic site in native gonococcal pili is inaccessible to antibody, or both.

Protein A is available commercially in a lyophilized, salt-free form ready for use with gold spheres. Large quantities of GpA complexes with a wide range of sphere size can be produced in the laboratory in half a day and can be stored for extended periods without loss of effectiveness. These characteristics make it possible to construct small gold spheres bearing antibodies against one microbial surface macromolecule and large gold spheres bearing antibodies against another surface macromolecule (10, 22, 28, 30). The use of both immunological probes in the same preparation (double label-

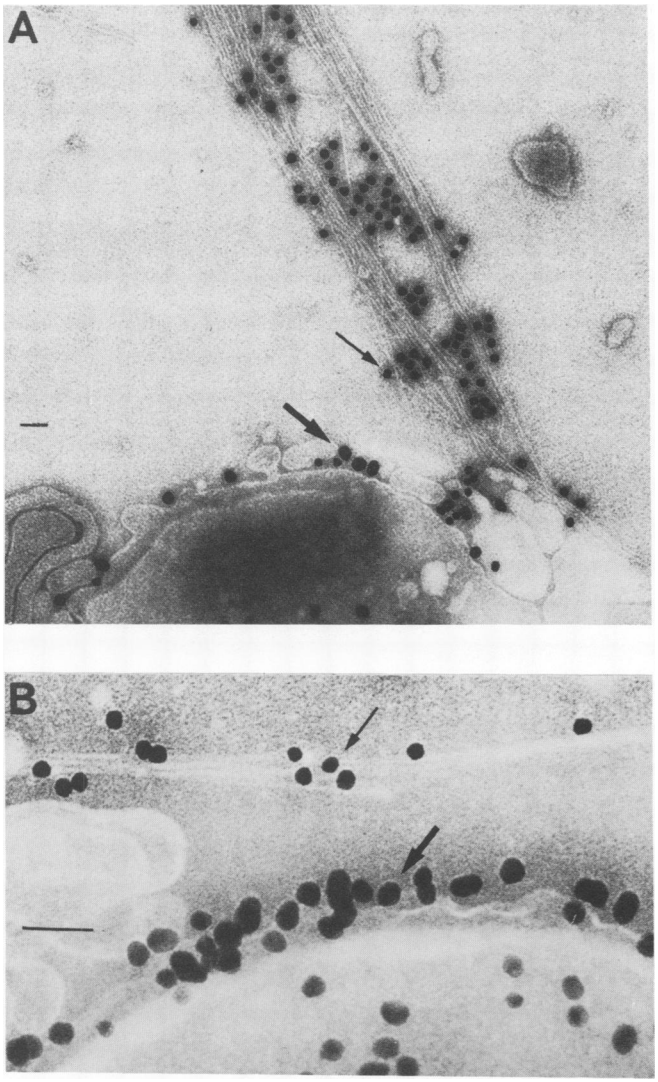

FIG. 2. Simultaneous localization of two antigenic structures. *N. gonorrhoeae* R10, a colony type 2 (piliated) transparent clone exposed sequentially to small gold spheres of 18-nm diameter conjugated to polyclonal anti-pilus antibody and large gold spheres of 40-nm diameter conjugated to monoclonal anti-protein I antibody. Note the attachment of small gold spheres to pili (small arrows), whereas large gold spheres are attached to the surface of the gonococcus (large arrow). Thus, the double-label technique allows the simultaneous localization of two antigens on the surface of viable microorganisms. Negative stain. Bars, 0.05 μm. Magnification: (A) ×64,000; (B) ×187,000.

ing) allows simultaneous localization of two different types of macromolecules.

A method that can localize antigens on the surface of intact microorganisms is increasingly important as purified proteins and protein fragments are tested as vaccines. Antibodies to macromolecules that are important in pathogenesis may fail to be protective if the epitopes against which they are directed are inaccessible—either buried beneath the surface of the outer membrane or enfolded in the tertiary structure of parent molecules. The method described here is an extremely useful tool to assess the ability of antibodies to reach their target epitopes when these are in a native state. In addition, the electron density of gold spheres allows them to be recognized easily in tissue sections. When coupled with antibody against macromolecules thought to be virulence factors (e.g., pili, lipopolysaccharide, outer membrane protein), gold spheres should allow the ultrastructural localization of those factors in naturally or experimentally infected tissues and aid in determining the role of these potential virulence factors in the disease process.

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